**CLAIMS:**

1. A method for producing a variant of a parent pullulanase, the variant having at least one altered property as compared to the parent pullulanase, the method comprising:

a) modeling the parent pullulanase on the three-dimensional structure of SEQ ID NO: 1 depicted in the Appendix to produce a three-dimensional structure of the parent pullulanase;

b) identifying in the three-dimensional structure obtained in step (a) at least one structural part of the parent pullulanase, wherein an alteration in said structural part is predicted to result in an altered property;

c) modifying the nucleic acid sequence encoding the parent pullulanase to produce a nucleic acid sequence encoding a deletion, insertion, or substitution of one or more amino acids at a position corresponding to said structural part; and

d) expressing the modified nucleic acid sequence in a host cell to produce the variant pullulanase.

2. The method according to claim 1, wherein the altered property is pH dependent activity, thermostability, substrate cleavage pattern, specific activity of cleavage, substrate specificity, such as higher isoamylase activity and/or substrate binding.

3. The method according to claim 2, wherein the altered property is a higher isoamylase activity as defined by an increase of at least 5% in the number of reducing ends formed in the "assay for isoamylase-like activity" described herein, using 50 mM sodium acetate, a pH of 4.5, 5.0 or 5.5, a temperature of 60°C and when incubated with a 10% w/v rabbit liver glycogen solution for a period of 10 min.

4. The method according to claims 1 or 2, wherein the altered property is an improved thermostability as defined by differential scanning calorimetry (DSC) using the method described herein.

5. The method according to claims 1 or 2, wherein the altered property is an improved thermostability as defined by an increased half-life ($T_{½}$) of at least about 5%, preferably, at least about 10%, more preferably at least about 15%, more preferably at least about 25%, most preferably at least about 50%, such as at least

about 100%, in the "$T_{½}$ assay for liquefaction" described herein, using a pH of 5.0 and a temperature of 95°C.

6. The method according to claims 1 or 2, wherein the altered property is an improved thermostability as defined by an increased residual enzyme activity of at least about 5%, preferably, at least about 10%, more preferably at least about 15%, more preferably at least about 25%, most preferably at least about 50%, such as at least about 100%, in the "assay for residual activity after liquefaction" described herein, using a pH of 5.0 and a temperature of 95°C.

7. The method according to claims 1 or 2, wherein the altered property is an improved thermostability as defined by an increased half-life ($T_{½}$) of at least about 5%, preferably, at least about 10%, more preferably at least about 15%, more preferably at least about 25%, most preferably at least about 50%, such as at least about 100%, in the "$T_{½}$ assay for saccharification" described herein, using a pH of 4.5 and a temperature of 70°C.

8. The method according to claims 1 or 2, wherein the altered property is an improved thermostability as defined by an increased residual enzyme activity of at least about 5%, preferably, at least about 10%, more preferably at least about 15%, more preferably at least about 25%, most preferably at least about 50%, such as at least about 100%, in the "assay for residual activity after saccharification" described herein, using a pH of 4.5 and a temperature of 63°C.

9. The method according to claim 8, wherein the "assay for activity for saccharification" described herein, is carried out at a pH of 4.5 and at a temperature of 70°C.

10. A method for constructing a variant of a parent pullulanase, the method comprising:

a) identifying an internal or external cavity or crevice in the three-dimensional structure of the parent pullulanase;

b) substituting at least one amino acid residue in the neighborhood of the cavity or crevice with another amino acid residue which increases the hydrophobic interaction and/or fills out or reduces the size of the cavity or crevice;

c) optionally repeating steps a) and b) recursively;

d) optionally making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than b);

e) preparing the variant resulting from steps a) - d);

f) testing the thermostability of said variant; and

g) optionally repeating steps a) - f) recursively; and

h) selecting a variant having increased thermostability as compared to the parent pullulanase.

11. A method for constructing a variant of a parent pullulanase, the method comprising:

a) identifying in the three-dimensional structure of the parent pullulanase two or more amino acid residues which, when substituted with cysteines, are capable of forming a disulfide bond;

b) substituting the amino acids identified in a) with cysteines;

c) optionally repeating steps a) and b) recursively;

d) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than b);

e) preparing the variant resulting from steps a) - d);

f) testing the thermostability of said variant; and

g) optionally repeating steps a) - f) recursively; and

h) selecting a variant having increased thermostability as compared to the parent pullulanase.

12. A method for constructing a variant of a parent pullulanase, the method comprising:

a) identifying, on the surface of the parent pullulanase, at least one amino acid residue selected from the group consisting of Asp, Glu, Arg, Lys and His;

b) substituting, on the surface of the parent pullulanase, at least one amino acid residue selected from the group consisting of Asp, Glu, Arg, Lys and His with an uncharged amino acid residue.

c) optionally repeating steps a) and b) recursively;

d) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than b);

e) preparing the variant resulting from steps a) - d);

f) testing the thermostability of said variant; and

g) optionally repeating steps a) - f) recursively; and

h) selecting a variant having increased thermostability as compared to the parent pullulanase.

13. A method for constructing a variant of a parent pullulanase, the method comprising:

a) identifying an amino acid sequence which links together two or more domains of the parent pullulanase together;

b) substituting at least one amino acid residue in the said amino acid sequence with another amino acid residue or deleting at least one amino acid residue in said amino acid sequence;

c) optionally repeating steps a) and b) recursively;

d) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than b);

e) preparing the variant resulting from steps a) - d);

f) testing the thermostability of said variant; and

g) optionally repeating steps a) - f) recursively; and

h) selecting a variant having increased thermostability as compared to the parent pullulanase.

14. A method according to claim 13, the method comprising step b) deleting at least one amino acid residue in said amino acid sequence;

15. A method according to any of claims 10-14, wherein the increased thermostability is as defined in any of claims 4-9.

16. A method for constructing a variant of a parent pullulanase, where the variant pullulanse has an altered substrate specificity as compared to the parent pullulanase, the method comprising:

a) identifying the substrate binding area in a model of the three-dimensional structure of the parent pullulanase;

b) modifying the substrate binding area by an amino acid substitution, deletion and/or insertion;

c) optionally repeating step b) recursively;

d) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than b),

e) preparing the variant resulting from steps a) - d);

f) testing the substrate specificity of the variant;

g) optionally repeating steps a) - f) recursively; and

h) selecting a variant having an altered substrate specificity as compared to the parent pullulanase.

17. The method according to claim 16, wherein the altered substrate specificity is an increased isoamylase activity compared to the parent pullulanase.

18. The method according to claim 17, wherein the increased isoamylase activity is defined by an increase of at least 5% in the number of reducing ends formed in the "assay for isoamylase-like activity" described herein, using 50 mM sodium acetate, a pH of 4.5, 5.0 or 5.5, a temperature of 60°C and when incubated with a 10% w/v rabbit liver glycogen solution for a period of 10 min.

19. A method for constructing a variant of a parent pullulanase, the method comprising:

a) ident an amino acid residue which is wi 5 Å, in particular 10 Å, from an active site residue of the parent pullulanase in the three-dimensional structure of said parent pullulanse, and which is involved in electrostatic or hydrophobic interactions with an active site residue;

b) substituting said amino acid residue with another amino acid residue which changes the electrostatic and/or hydrophobic surroundings of an active site residue, and which can be accommodated in the structure;

c) optionally repeating steps a) and b) recursively;

d) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than b);

e) preparing the variant resulting from steps a) - d);

f) testing the pH dependent activity of said variant; and

g) optionally repeating steps a) - f) recursively; and

h) selecting a variant having an altered pH dependent activity as compared to the parent amylase.

20. A method according to any of the preceding claims, wherein the parent pullulanase has more than 40% homology with the amino acid sequence shown in SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5, preferably more than 50%, such as more than 60%, more than 70%, more than 75%, more than 80%, more than 85%, more than 90%, more than 91%, more than 92%, more than 93%, more than 94%, more than 95%, more than 96%, more than 97%, more than 98%, more than 99% homology with the amino acid sequence shown in SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5.

21. A method according to claim 20, wherein the parent pullulanase has the amino acid sequences shown in SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5.

22. A method for producing a pullulanase variant, the method comprising:

a) constructing the variant by the method according to any of claims 10-21;

b) transforming a microorganism with a DNA sequence encoding the variant;

c) cultivating the transformed microorganism under conditions which are conducive for producing the variant; and

d) optionally, recovering the variant from the resulting culture broth.

23. A pullulanase variant, wherein

(a) the variant has more than 40% homology to SEQ ID NO:1;

(b) the variant comprises an amino acid modification compared to SEQ ID NO:1 in at least one of the positions corresponding to 95-113, K122P, 130-140, K151P, 157-165, 180, 181, 210, 227, 228, 232-238, 259, 266-272, 286, G293P, 298, 299, 300-314, such as 302-308, N315P, 337-339, 353, N374P, 380, 384, 385, 392, 394, 396, 406, 408-429, such as 418-428, 442, A446P, 478, 500-507, 515, 526, 534, 543, 544 550, T556P, 557, 563, 568, 573, 576, 583, 627, 659-665, G668P, G672P, 681, 684, 688, 689, 751-755, 732, 736, 740, 760, 767, 770 783, 788, 792, 793, such as N793P, K758C+I914C, T916C+A765C, I897C+S819C, P525C+E499C and H286C+T148C;

(c) the variant has an improved thermostability as compared to the parent pullulanase.

24. A pullulanase variant, wherein

(a) the variant has more than 40% homology to SEQ ID NO:1;

(b) the variant comprises an amino acid modification compared to SEQ ID NO:1 in at least one of the positions corresponding to 437, 439, 487, 489, 490, 494-496, 505-511, 514, 551-559, 584-590, 620-626, 650-658, 665-668, 679, 681, 684, 685, 690-693, 731, 734-738, 775, 786, and 789-795;

(c) the variant has an increased isoamylase activity as compared to the parent pullulanase.

25. A pullulanase variant, wherein

(a) the variant has more than 40% homology to SEQ ID NO:1;

(b) the variant comprises an amino acid modification compared to SEQ ID NO: 1 in at least one of the positions corresponding to 430, 433, 435-443, 486-496, 505-515, 518, 521, 548-560, 565, 573-575, 583-595, 599, 600,

602-606-608, 610, 611, 616-633, 635, 639, 646-672, 674-696, 717, 720-722, 725-747, 760, 763, 764, 767, 773-781, 783-797, 799-802 and 817;

(c) the variant has an altered pH dependent activity as compared to the parent pullulanase.

26. A pullulanase variant according to any of claims 23, 24 or 25, wherein the variant has more than 45% homology with the amino acid sequence shown in SEQ ID NO: 1, preferably more than 50%, such as more than 60%, more than 70%, more than 75%, more than 80%, more than 85%, more than 90%, more than 91%, more than 92%, more than 93%, more than 94%, more than 95%, more than 96%, more than 97%, more than 98%, more than 99% homology with the amino acid sequence shown in SEQ ID NO: 1.

27. A pullulanase variant according to claim 26, wherein the parent pullulanase has the amino acid sequence shown in SEQ ID NO:1.

28. A pullulanase variant, wherein

(a) the variant has more than 40% homology to SEQ ID NO:3;

(b) the variant comprises an amino acid modification compared to SEQ ID NO:3 in at least one of the positions corresponding to 111, 112, 158-160, 270-274, 302-314, 392, 400, 408-426, 428, 437, 440, 444, 446, 483, 485, 487, 492, 495, 504, 551, D526P, 530, 543, 566, 613, 621, 710, 717, 735, 775, 779, 789, G794P, 823, 855, 891, 892, 437+503 and 674+664;

(c) the variant has an improved thermostability as compared to the parent pullulanase.

29. A pullulanase variant, wherein

(a) the variant has more than 40% homology to SEQ ID NO:3;

(b) the variant comprises an amino acid modification compared to SEQ ID NO:3 in at least one of the positions corresponding to 435, 437, 485, 487, 488, 492-494, 503-509, 512, 549-557, 582-588, 618-624, 648-656, 663-666, 677, 679, 682, 683, 688-691, 729, 732-736, 773, 784, 787-793;

(c) the variant has an increased isoamylase activity compared to the parent pullulanase.

30. A pullulanase variant, wherein

(a) the variant has more than 40% homology to SEQ ID NO:3;

(b) the variant comprises an amino acid modification compared to SEQ ID NO: 3 in at least one of the positions corresponding to 428, 431, 433-441, 484-494, 503-513, 516, 519, 546-558, 563, 571-573, 581-593, 597, 598, 600-602, 604-606, 608, 609, 614-631, 633, 634, 637, 644-670, 672-694, 715, 718-720, 723-745, 758, 761, 762, 765, 771-779, 781-795, 797-800, and 815;

(c) (c) the variant has an altered pH dependent activity as compared to the parent pullulanase.

31. A pullulanase variant according to any of claims 28, 29 or 30, wherein the variant has more than 45% homology with the amino acid sequence shown in SEQ ID NO: 3, preferably more than 50%, such as more than 60%, more than 70%, more than 75%, more than 80%, more than 85%, more than 90%, more than 91%, more than 92%, more than 93%, more than 94%, more than 95%, more than 96%, more than 97%, more than 98%, more than 99% homology with the amino acid sequence shown in SEQ ID NO: 3.

32. A pullulanase variant according to claim 31, wherein the parent pullulanase has the amino acid sequence shown in SEQ ID NO:3.

33. A variant according to claims 23 or 28, wherein the improved thermostability is defined by an increased half-life ($T_{½}$) of at least about 5%, preferably, at least about 10%, more preferably at least about 15%, more preferably at least about 25%, most preferably at least about 50%, such as at least about 100%, in the "$T_{½}$ assay for liquefaction" described herein, using a pH of 5.0 and a temperature of 95°C.

34. A variant according to claims 23 or 28, wherein the improved thermostability is defined by an increased residual enzyme activity of at least about 5%, preferably, at least about 10%, more preferably at least about 15%, more preferably at least about 25%, most preferably at least about 50%, such as at least about 100%, in the "assay

for residual activity after liquefaction" described herein, using a pH of 5.0 and a temperature of 95°C.

35. A variant according to claims 23 or 28, wherein the improved thermostability is defined by an increased half-life ($T_{½}$) of at least about 5%, preferably, at least about 10%, more preferably at least about 15%, more preferably at least about 25%, most preferably at least about 50%, such as at least about 100%, in the "$T_{½}$ assay for saccharification" described herein, using a pH of 4.5 and a temperature of 70°C.

36. A variant according to claims 23 or 28, wherein the improved thermostability is defined by an increased residual enzyme activity of at least about 5%, preferably, at least about 10%, more preferably at least about 15%, more preferably at least about 25%, most preferably at least about 50%, such as at least about 100%, in the "assay for residual activity after saccharification" described herein, using a pH of 4.5 and a temperature of 63°C.

37. A variant according to claim 36, wherein the "assay for activity for saccharification" described herein, is carried out at a pH of 4.5 and at a temperature of 70°C.

38. A variant according to claims 24 or 29, wherein the increased isoamylase activity is defined by an increase of at least 5% in the number of reducing ends formed in the "assay for isoamylase-like activity" described herein, using 50 mM sodium acetate, a pH of 4.5, 5.0 or ~~5.5,~~ a temperature of 60°C and when incubated with a 10% w/v rabbit liver glycogen solution for a period of 10 min.

39. A variant according to any of claims 23, 25, 28 or 30, wherein the variant further has an increased isoamylase activity as compared to the parent pullulanase.

40. A variant according to claim 37, wherein the increased isoamylase activity is as defined in claim 38.

41. A variant according to any of claims 24, 25, 28 or 30, wherein the variant further has an improved thermostability as compared to the parent pullulanase.

42. A variant according to claim 41, wherein the improved thermostability is as defined in any of claims 33-37.

43. A variant according to any of claims 23, 24, 28 or 29, wherein the variant further has an altered pH dependent activity as compared to the parent pullulanase.

44. An isolated nucleic acid sequence comprising a nucleic acid sequence, which encodes for the pullulanase variant defined in any of claims 23-43.

45. An isolated nucleic acid sequence according to claim 44, wherein the nucleic acid sequence is selected form the group consisting of:

(a) a nucleic acid sequence having at least 40% homology with the nucleic acid sequence shown in SEQ ID NO: 1 or SEQ ID NO: 3, and

(b) a nucleic acid sequnce which hybridizes under low stringency conditions, preferably under medium stringency conditions, in particular under high stringency conditions, with

(i) a complementary strand of the nucleic acid sequence shown in SEQ ID NO: 1 or SEQ ID NO: 3, or

(ii) a subsequence of (i) of at least 100 nucleotides.

46. An isolated nucleic acid sequence according to claim 45, wherein the nucleic acid sequence has at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% homology with the nucleic acid sequence shown as SEQ ID NO: 1 or SEQ ID NO:3.

47. An isolated nucleic acid construct comprising a nucleic acid sequence as defined in any of claims 44-46, operably linked to one or more control sequences capable of directing the expression of the polypeptide in a suitable expression host.

48. A recombinant expression vector comprising the nucleic acid sequence of claim 47, a promoter, and transcriptional and translational stop signals, and preferably further comprising a selectable marker.

49. A recombinant host cell comprising the nucleic acid construct of claim 47.

50. A method for producing the variant defined in any of claims 23-43, the method comprising:

(a) cultivating the recombinant host cell of claim 49 under conditions conducive to the production of the pullulanase variant; and

(b) recovering the variant.

51. A method for converting starch to one or more sugars, the method comprising debranching the starch using at least one pullulanase obtained by the methods defined in any of claims 1-22.

52. A method for converting starch to one or more sugars, the method comprising debranching the starch using at least one pullulanase variant as defined in any of claims 23-44.

53. An isolated nucleic acid sequence comprising the nucleic acid sequence shown in SEQ ID NO: 1.

54. A nucleic acid sequence according to claim 53, wherein the isolated nucleic acid sequence consists of the nucleic acid sequence shown in SEQ ID NO: 1.

55. An isolated nucleic acid construct comprising a nucleic acid sequence as defined in claims 53 or 54, operably linked to one or more control sequences capable of directing the expression of the polypeptide in a suitable expression host.

56. A recombinant expression vector comprising the nucleic acid sequence of claim 55, a promoter, and transcriptional and translational stop signals, and preferably further comprising a selectable marker.

57. A recombinant host cell comprising the nucleic acid construct of claim 55.



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